# METHOD FOR AMPLIFICATION OF RNA SEQUENCES

The present invention relates to a method for amplification of target RNA sequences, to primers for the
5 amplification of target RNA sequences and to a kit comprising one or more of the primers.

Nucleic acid amplification methods, wherein target RNA or DNA sequences are amplified, are generally applied in the field of molecular biology, biochemistry and
10 biotechnology and the possible applications are numerous and still increasing. The amplification methods increase the number of copies of a particular target nucleic acid sequence, which often is present in small amounts and in an environment in which a wide variety of other nucleic acids,
15 both RNA and DNA, are present.

Nucleic acid amplification methods are in particular used to facilitate the detection and quantification of specific target nucleic acid sequences present in samples such as blood, sperm, tissue, hair, microorganisms, cells,
20 tumors etc., in diagnosing, for example, infectious diseases, inherited diseases, and various types of cancer. In addition, nucleic acid amplification methods have found their applications in other fields where samples are investigated in which informative target nucleic acids may be present in
25 minute amounts, such as in forensic sciences and in archeology or to establish paternity.

Several nucleic acid amplification techniques are known, like Polymerase Chain Reaction (PCR) and Transcription Based Amplification (TBA), which are based on different
30 mechanisms of action, but all depend on the annealing of one or more primers to the boundaries of the target nucleic acid sequence or its complementary sequence. The annealing of a primer to the boundary of the target nucleic acid sequence is provided by the hybridization of a contiguous number of

nucleotides comprised in the primer, to a contiguous number
of complementary nucleotides in the target nucleic acid
sequence.

     Efficient hybridization of the primer generally

5  requires at least 15 contiguous complementary nucleotides. If
less nucleotides are present, the ratio between specific and
non-specific annealing of the primer decreases below a point
where no longer effective amplification of the target nucleic
acid can occur. The requirement of at least 15 contiguous

10  complementary nucleotides, however, limits the possibility of
for example amplification of target nucleic acids in case
only a consensus sequence of the target sequence can be used,
especially in cases when all genotypes from target nucleic
acids derived from organisms like viruses, bacteria, and

15  yeast, or from tissue specimens like blood, cells, tumors,
and lymphoid fluids are to be amplified. When, due to genetic
variation, the consensus sequence may vary among individual
species, inefficient hybridization of the primers as a
consequence of the presence of non-complementary nucleotides

20  may occur.

     If several "genetic variations" of a target
nucleotide sequence are known, conserved stretches of
nucleotides can be identified and used for primer design.
However, preferred conserved stretches in some species, like

25  RNA viruses, such as parts of the coding region of the human
hepatitis C virus, are sometimes too small to be used to
design efficient primers. In addition, even these conserved
segments have a tendency to mutate, which is most pronounced
in fast dividing organisms like viruses and bacteria,

30  resulting in a reduced efficiency of hybridization of the
primer. As a consequence, amplification may not occur, which
is highly undesirable in for example routine diagnostics

since a false negative amplification result can have severe
consequences for the patient concerned.

Therefore there is a clear need for an amplification
method which allows for amplification using small stretches
5   of target RNA sequences and which method is less prone to
mutations or genetic variations in the hybridizing segment of
the target sequence.

In the research that led to the present invention it
has been found that the above-mentioned problems can be
10  obviated by an amplification method comprising the steps of:

(a) annealing a first primer to the target RNA
sequence, said first primer comprising a hybridizing
sequence, which is complementary to and hybridizes to
at least a first segment of the target RNA sequence,
15          operatively associated with a promoter sequence;

(b) extending said first primer in a reaction
catalyzed by a DNA polymerase, forming a first
RNA/cDNA hybrid nucleic acid molecule;

(c) selectively removing the target RNA sequence of
20          the first RNA/cDNA hybrid nucleic acid molecule
forming a first single stranded cDNA sequence;

(d) annealing a second primer to the obtained first
single stranded cDNA sequence, said second primer
comprising a hybridizing sequence which is
25          complementary to and hybridizes to a first segment of
the first single stranded cDNA sequence;

(e) extending said second primer in a reaction
catalyzed by a DNA polymerase to form a first double
stranded DNA molecule; and

30  (f) employing the first double stranded DNA molecule
of step (e) in the preparation of a plurality of RNA
transcripts that are complementary to the target RNA
sequence in a reaction catalyzed by a DNA-dependent

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RNA polymerase with specificity for the promoter
sequence comprised in the first primer;

wherein the first primer comprises a hybridizing
sequence of 7 to 14 nucleotides, a transcription enhancing
5    sequence, and an anchor which is capable of binding to a
second segment of the target RNA sequence, and/or wherein the
second primer comprises a hybridizing sequence of 7 to 14
nucleotides, an amplification enhancing sequence and an
anchor which is capable of binding to a second segment of the
10   first single stranded cDNA.

According to the present invention it has
surprisingly been found that a reduction of the number of
contiguous hybridizing nucleotides in at least one of the
primers still allows for a reliable amplification method if
15   said primer additionally comprises an anchor which binds to a
second segment of the target RNA sequence, which is not the
hybridizing nucleotide sequence, in such a way that the
anchor exposes the hybridising sequence to the first segment.

The use of an anchor in addition to a hybridizing
20   sequence thus results in a two-step annealing process:
1) binding of the anchor to a segment of the target RNA
sequence, designated as the second segment, and 2)
hybridization of the hybridizing sequence of the primer to
the contiguous complementary nucleotides of the target RNA
25   sequence, designated as the first segment, which steps can
occur simultaneously or sequentially (i.e. first step 1 and
then step 2 or first step 2 and then step 1). The anchor may
bind specifically, but the anchor and hybridizing sequence
may also together be responsible for the specific interaction
30   of the primer to the target RNA, for example in case the
anchor is 7-14 nucleotides in length and the hybridizing
sequence is 13-7 nucleotides in length, by which the anchor

and hybridizing sequence independently do not bind
specifically, but the combination is specific.

        According to the invention, the number of nucleotides
in the hybridizing sequence of the primers can be reduced to
5   14, 13, 12, 11, 10, 9, 8, or 7 nucleotides. Due to the
presence of the anchor in the primer, specific binding of the
primer to the target nucleotide sequence will occur,
regardless of the fact that the hybridizing part only
contains maximally 14 nucleotides, and in itself would not be
10  able to hybridize specifically. Thus, by the combination of
the anchor and the hybridizing sequence specific binding of
the primer to the target sequence is accomplished.

        The method according to the invention is further
explained referring to Figure 16, wherein a preferred
15  embodiment of the method is shown. During the first step the
PNA anchor present in the first primer (P1), usually
designated as the forward primer, binds to a segment of the
target RNA sequence (RNA+). Subsequently, the hybridizing
sequence (H) of the first primer hybridizes to the first
20  segment of the target RNA sequence.

        After annealing of the first primer to the target RNA
sequence, the primer is extended using a DNA polymerase, such
as the avian myeloblastosis virus (AMV) reverse transcriptase
polymerase. The DNA polymerase will copy the target RNA
25  sequence forming a first RNA/cDNA hybrid molecule.

        Simultaneously, or subsequently the target RNA
sequence is selectively removed, preferably by enzymatic
digestion with for example an RNase. The resulting first
single stranded cDNA sequence (DNA-) is used as a template
30  for the subsequent step in the amplification process.

        This subsequent step requires the annealing of a
second primer (P2), usually designated the reverse primer, to
the formed first single stranded cDNA. According to the shown

embodiment, the second primer also comprises a hybridizing
sequence (H) which is complementary to and hybridizes to a
first segment of the single stranded cDNA, an amplification
enhancing sequence (X) and a PNA anchor which is capable of
specific binding to a second segment of the first single
stranded cDNA sequence.

According to the present invention, both the first
and second primer, or only the first or second primer, may
comprise a short hybridizing sequence and an anchor.

In a subsequent step of the amplification method, the
second primer is extended by the AMV reverse transcriptase
polymerase using the first single stranded cDNA sequence as a
template resulting in a first double stranded DNA molecule
including a double stranded promotor sequence derived from
the first primer.

This first double stranded DNA molecule is
subsequently used for the preparation of a plurality of RNA
transcripts (RNA-) by a DNA-dependent RNA polymerase. The
sequence downstream of the promotor site is used as template
meaning that the RNA transcripts will not include the anchor
sequence of the forward primer.

It will be obvious for the person skilled in the art
that the choice of the DNA-dependent RNA polymerase is
dependent on the choice of the promoter sequence in the first
primer. In a preferred embodiment of the present invention,
the promoter sequence is the T7 promoter sequence and the
DNA-dependent RNA polymerase is the T7 polymerase.

In a further preferred embodiment, the method
according to the invention further comprises the following
steps:

   (g) annealing of the second primer to the RNA
       transcripts produced in step (f);

(h) extending the second primer in a reaction
catalyzed by the DNA polymerase to form a second
RNA/cDNA hybrid nucleic acid molecule;

(i) selectively removing the RNA of the second
5       RNA/cDNA hybrid molecule to obtain a second single
stranded cDNA molecule;

(j) annealing the first primer to the obtained second
single stranded cDNA sequence;

(k) extending the 3' end of the second single
10      stranded cDNA molecule in a reaction catalyzed by the
DNA polymerase using the first primer as a template
to form a second partly double stranded DNA molecule
comprising a double stranded promotor site;

(l) employing the second partly double stranded DNA
15      molecule of step (k) in the preparation of a
plurality of RNA transcripts complementary to the
target RNA sequence in a reaction catalyzed by the
DNA-dependent RNA polymerase with specificity for the
promotor sequence in the first primer.

20      These additional steps of the amplification method
according to the present invention result in a self-sustained
(or cyclic) amplification of the target RNA sequence, which
is also schematically represented in Figure 16B.

In a preferred embodiment of the present invention,
25  the first primer comprises, going from the 5' end to the 3'
end, an anchor, a transcription enhancing sequence, and a
hybridizing sequence consisting of 7 to 14 nucleotides which
are complementary to a first segment of the target RNA
sequence of 7 to 14 contiguous nucleotides.

30      In a further preferred embodiment, the second primer
comprises of, going from the 5' end to the 3' end, an anchor,
an amplification enhancing sequence, and a hybridizing
sequence consisting of 7 to 14 nucleotides which are

complementary to a the first segment of the first single
stranded cDNA sequence of 7 to 14 contiguous nucleotides.

Preferably, the hybridizing sequence of the first and
second primer comprises 7 to 10 nucleotides which are

5   complementary to a the first segment of the RNA target
sequence or the first single stranded cDNA sequence of 7 to
10 contiguous nucleotides, respectively.

Due to the presence of the anchor, the primer is able
to bind specifically to the target RNA sequence. The

10  hybridizing sequence alone would not be able to bind
specifically, due to its short length.

According to the invention, the anchor can be any
compound which is capable of binding to a second segment of
the target RNA sequence. The anchor preferably is an,

15  optionally modified, oligonucleotide or a protein. Preferred
is an, optionally modified, oligonucleotide which comprises
7-22, optionally modified, nucleotides, more preferably 7-14,
most preferably 9-14 nucleotides, whichs bind to the second
segment of the target RNA sequence or the second segment of

20  the single stranded cDNA sequence. The person skilled in the
art will understand that for binding of the anchor it is not
required that all nucleotides in the anchor are complementary
to the corresponding sequence in the second segment of the
target RNA sequence. Thus, for specific binding of the primer

25  to the target RNA sequence approximately at least 90% of the
nucleotides of the anchor should be complementary. This
allows for a more flexible selection of the second segment
without influencing the efficiency of the amplification
process.

30      The nucleotides of the anchor can be both RNA and
DNA, or modified nucleotides, such as locked nucleic acids
(LNA) or 2'-O-methyl modified nucleotides, or peptide nucleic
acids (PNA). Such modifications provide either a more stable

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anchor or enhance the specific binding characteristics of the
primer.

Proteins, or fragments derived thereof, which display
specific binding to nucleic acid sequences can also be used
5   as the anchor. Proteins, or fragments derived thereof, which
are capable of binding to the second segment of the target
RNA sequence or the second segment of the first single
stranded cDNA sequence can be selected or designed by for
example protein engineering. Examples of such proteins are
10  polyC-binding proteins, polyA-binding proteins, proteins
comprising one or more zinc-finger, restriction enzymes,
and/or antibodies and fragments thereof such as scFvs, Fabs
etc.

The first and second segment of the target nucleic
15  acid sequence preferably are separated by 0, 1, 2, 3, 4, 5 or
6 nucleotides, more preferably by 0, 1, 2, 3 or 4
nucleotides, and most preferably by 0, 1, 2 or 3 nucleotides.
When more than 6 nucleotides are present between the two
segments, the efficiency of the two-step annealing of the
20  primer and thus of the amplification is reduced.

The single stranded RNA transcripts produced during
amplification are extremely suitable for the use of
hybridization-based detection systems with sequence-specific
probes. The detection may take place at the end of the
25  amplification using electro-chemiluminescence (ECL). A
specific capture probe, attached to magnetic beads via
streptavidin-biotin interaction, is used to immobilize the
RNA transcript and a ruthenium-labeled detection probe
hybridizes with the transcript. Also other currently known
30  detecion methods may be used, such as for example "enzyme
linked gel assay" (ELGA) or fluorescence spectroscopy.

A very elegant detection method is the use of
molecular beacons. Molecular beacons are DNA oligonucleotides

labeled with a fluorophore at the 5'-end. The last part of
the 3'-end of the sequence is complementary to the first part
of the 5'-end and a hairpin stem is formed in such as way
that a quencher at the 3'-end absorbs the emitted light of

5   the fluorophore. The hairpin loop sequence is complementary
to the target sequence of the RNA transcript. Because of the
binding of the loop sequence to the target sequence, the
hairpin stem opens up and the quencher becomes separated from
the fluorophore. The increase in light emitted can be

10  detected by a fluorometer. Hybridization with the RNA
transcript takes place during amplification enabling "real-
time" detection. To quantify the amount of target nucleic
acid in a sample, a calibrator may be included, which is
added prior to the isolation of the nucleic acid and is co-

15  extracted and co-amplified with the target nucleic acid.
        The detection probe may be a target-specific probe or
may be a generic probe, i.e. directed against a generic
sequence incorporated in the RNA transcript, such as a
sequence identical to the amplification enhancing sequence.

20      Thus, in a preferred embodiment of the method
according to the invention, the target-specific probe
hybridizes to the RNA transcript, complementary to the target
RNA sequence. According to another preferred embodiment, the
generic probe hybridizes to the sequence identical to the

25  amplification sequence of the second primer, enabling the use
of one sequence-specific probe for the detection of a wide
variety of different amplified target RNA sequences. This
results in a cost effective detection method (detection
probes are in general relatively expensive to synthesize),

30  and increases the chances for reproducible and quantitative
detection, since the binding characteristics of the probe
could be comparable in every amplification method regardless
of the starting materials or the target RNA sequence.

The amplification method of the present invention is preferably used for the amplification of viral target RNA sequences, such as RNA isolated from the human immunodeficiency virus (HIV) or RNA isolated from the human
5    hepatitis C virus, which viruses have only partially conserved nucleic acid sequences.

The invention further relates to primers comprising an anchor and a hybridizing sequence. The primers according to the invention preferably comprise, going from the 5' end
10   to the 3' end, an anchor, as defined above, a transcription enhancing sequence or an amplification enhancing sequence, as defined below, and a hybridizing sequence of 7 to 14 nucleotides, preferably 7 to 10 nucleotides, as defined above.

15      The use of the primers comprising an anchor and a hybridizing sequence is not limited to Transcription Based Amplification (TBA), but these primers can also be used in other amplification methods like the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

20      According to another aspect of the present invention, the invention relates to a kit for the amplification and/or detection of a target RNA sequence comprising at least one or more primers according to the invention.

The kit according to the invention may further
25   comprise one or more sequence-specific probes, an amplification buffer, and/or one or more enzymes, such as a DNA polymerase, RNAse H, and a DNA-dependent RNA polymerase.

DEFINITIONS

30      According to the present invention a target RNA sequence is defined as a specific segment of RNA which is to be amplified by an amplification method.

Hybridization is defined as the binding of a contiguous number of nucleotides, present in for example a primer, to the complementary nucleotides of for example a target RNA sequence. The hybridization results in the
5    formation of a double stranded nucleic acid molecule.

Annealing is defined as the association of a primer with a target nucleic acid sequence by for example hybridization.

Specific binding is defined as the preference for
10   binding of a compound (such as a primer, or part of a primer) to a specific oligonucleotide sequence, as compared to the binding of said compound to a random nucleotide sequence under the used conditions. Thus a compound binds specifically if the compound only binds to one and the same nucleotide
15   sequence.

A nucleic acid sequence is designated complementary if all individual nucleotides of the nucleic acid sequence are complementary, for example, a oligonucleotide sequence reads 5'-ATG ACC TGG-3' and the complementary oligonucleotide
20   sequence reads 3'-TAC TGG ACC-5'.

A template is defined as a nucleic acid molecule that is to be transcribed by a polymerase. The synthesized transcript is complementary to the nucleic acid molecule, or at least to one strand of the nucleic acid molecule. Both RNA
25   and DNA are usually synthesized in the 5' to 3' direction.

A transcription enhancing sequence is a nucleic acid sequence comprising a promotor, for example T7, which is a non-specific sequence vis-à-vis the target sequence. The transcription enhancing sequence may optionally comprise a
30   purine stretch (mainly composed by G and/or A). It creates a loop between the anchor and the hybridizing sequence.

An amplification enhancing sequence is a non-specific nucleic acid vis-à-vis the target sequence, but it comprises

no promotor, i.e. only a random sequence that generates a
loop between the anchor and the hybridizing sequence.

5       The invention will be further exemplified by the
following Figures and Examples. These Figures and Examples
are not intended to limit the present invention in any way.


**FIGURES**

        Figure 1 shows the legend used in the graphs of the
subsequent figures.

10      Figure 2: Amplification of HIV RNA (RNA transcript,
500, 50 and 5 cps) with an anchor p1 primer (Fig. 2B: HIV12,
table 1) or a standard p1 primer (Fig. 2A: HIV1, table 1),
both in combination with a standard p2 primer (HIV2, table 1)
and a molecular beacon (HIV-MB-WT, table 1) as probe. A
15      sample without template (NT) is used as negative control.

        Figure 3: Schematic representation of anchor p1
primer HIV12 (table 1) and primer HIV11 (table 1) without
anchor.

        Figure 4: Amplification of HIV RNA (RNA transcript,
20      500, 50 and 5 cps) with a standard p1 primer (Fig. 4A: HIV1,
table 1), an anchor p1 primer (Fig. 4C: HIV12, table 1), a p1
primer without anchor (Fig. 4B: HIV11, table 1) or a
combination of primer HIV12 and HIV11 (Fig. 4D). All in
combination with a standard p2 primer (HIV2, table 1) and a
25      molecular beacon (HIV-MB-WT, table 1) as probe. A sample
without template (NT) is used as negative control.

        Figure 5: Amplification of HIV RNA (Total viral RNA,
500, 50 and 5 cps) with a standard p1 primer (Fig. 5A: HIV1,
table 1), anchor primers with different anchor lengths
30      varying from 22 to 7 nucleotides (HIV12 (Fig. 5B), 17 (Fig.
5C), 20 (Fig. 5D), 21 (Fig. 5E), 22 (Fig. 5F), table 1). All
in combination with a standard p2 primer (HIV2, table 1) and

a molecular beacon (HIV-MB-WT, table 1) as probe. A sample
without template (NT) is used as negative control.

Figure 6-8: Amplification of HIV RNA (Total viral
RNA, 500, 50 and 5 cps) with anchor primers with different
5   anchor lengths varying from 14 to 9 nucleotides (HIV17 (Fig.
6A), 20 (Fig. 7A), 21 (Fig. 8A), table 1) and the same
primers containing anchors with 2'-O-Methyl nucleotides
(HIV17 MET (Fig. 6B), HIV20 METa & b (Fig. 7B and C), HIV21
METa & b (Fig. 8B and C), table 1). All in combination with a
10  standard p2 primer (HIV2) and a molecular beacon (HIV-MB-WT,
table 1) as probe. A sample without template (NT) is used as
negative control.

Figure 9: Amplification of HIV RNA (Total viral RNA,
500, 50 and 5 cps) with a standard p1 primer (HIV1, table 1),
15  anchor primers (HIV17 and HIV22, table 1) and p1 primers
containing anchors with LNA nucleotides (HIV22 LNA1-4, table
1). All in combination with a standard p2 primer (HIV2, table
1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A
sample without template (NT) is used as negative control.
20  Fig. 9A: standard P1 primer HIV1; Fig. 9B: anchor primer
HIV22 LNA1; Fig. 9C: anchor primer HIV22 LNA2; Fig. 9D:
anchor primer HIV17; Fig. 9E: anchor primer HIV22; Fig. 9F:
anchor primer HIV22 LNA3; Fig. 9G: anchor primer HIV22 LNA4.

Figure 10: Amplification of HIV RNA (Total viral RNA,
25  500, 50 and 5 cps) with anchor primers (Fig. 10A: HIV17 and
Fig. 10C: HIV21, table 1) and p1 primers containing PNA
anchors (Fig. 10B: HIV17 PNA and Fig. 10D: HIV21 PNA, table
1). All in combination with a standard p2 primer (HIV2, table
1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A
30  sample without template (NT) is used as negative control.

Figure 11: Amplification of HIV RNA (Total viral RNA,
500, 50 and 5 cps) with standard p2 primers (HIV2 (Fig. 11A)
and HIV26 (Fig. 11B), table 1) and anchor p2 primers (HIV27

(Fig. 11C) and HIV29 (Fig. 11D), table 1). All in combination with a standard p1 primer (HIV1, table 1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

5      Figure 12: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with anchor p2 primers with different anchor lengths varying from 22 to 14 nucleotides (HIV27 (Fig. 12A), 31 (Fig. 12C), 32 (Fig. 12D), 33 (Fig. 12E), 29 (Fig. 12B), table 1). All in combination with a standard p1 primer
10    (HIV1, table 1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

       Figure 13: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with an anchor p1 primer (HIV17, table 1)
15    in combination with a standard p2 primer (HIV2, table 1) (Fig. 13B or anchor p2 primers (HIV27 (Fig. 13C) and HIV29 (Fig. 13D), table 1). The standard primer set (HIV1/ HIV2, table 1) is used as a reference (Fig. 13A). All in combination with a molecular beacon (HIV-MB-WT, table 1) as
20    probe. A sample without template (NT) is used as negative control.

       Figure 14: Amplification of HCV RNA (RNA transcript, $5 \times 10^5$, $5 \times 10^4$ and $5 \times 10^3$ cps) with an anchor p1 primer (HCV13 (Fig. 14B) or HCV 46 (Fig. 14C), table 2) or a
25    standard p1 primer (HCV1 (Fig. 14A), table 2), both in combination with a standard p2 primer (HCV2, table 2) and a molecular beacon (HCV-WT3, table 2) as probe. A sample without template (NT) is used as negative control.

       Figure 15: Amplification of HCV RNA (RNA transcript,
30    $5 \times 10^6$ and $5 \times 10^5$ cps) with an anchor p1 primer (HCV22 (Fig. 15B), table 2), having a 3 nt gap between the anchor sequence and the 3' hybridizing sequence. Anchor p1 primer HCV13 (Fig. 14A)(table 2) is used as reference. Both anchored

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primers are combined with the standard p2 primer (HCV2, table
2) and molecular beacon (HCV-WT3, table 2). A sample without
template (NT) is used as negative control.

Figure 16: Scheme showing a preferred embodiment of
5    the method according to the invention. P1: first primer; T:
transcription enhancing sequence; T7: T7 promotor sequence,
H: hybridizing sequence; P2: second primer; X: amplification
enhancing sequence; H: hybridizing sequence.


10   **EXAMPLES**


**EXAMPLE 1**


Amplification of HIV RNA with anchor p1 primer
15         Scott Layne HIV particles (HIV-infected cells (Layne
et al (1992), Virology 189: 695-714) lysed with lysisbuffer
(NucliSens, BioMérieux)) or HIV gag RNA transcript were used
as input material for amplification. Inputs of 500, 50 and 5
cps were tested. Amplification was performed in NASBA buffer
20   (40 mM Tris-HCl pH 8.5, 12 mM $MgCl_2$, 70 mM KCl, 15% v/v DMSO,
5 mM DTT, 1 mM each dNTP, 2 mM ATP, 2 mM CTP, 2 mM UTP, 1.5
mM GTP, 0.5 mM ITP, 0.2 µM of each primer (forward primer
(P1) and reverse primer (P2), table 1), 0.1 µM molecular
beacon probe (HIV-MB-WT, table 1). The mixture was incubated
25   for 2 min at 65 °C to denature the RNA and for 2 min at 41°C,
to hybridize the P1 primer to the target. Subsequently, NASBA
enzymes (0.08 units RNase H, 32 units T7 RNA polymerase, 6.4
units AMV reverse transcriptase and 2.1 µg BSA) were added,
the reaction mixture was mixed by gently tapping and short
30   centrifugation, and the amplification and real-time detection
was started. The reaction mixture was incubated at 41˙C in
the NucliSens EasyQ Analyzer (NucliSens, BioMérieux) for 60
minutes with fluorescence monitoring every minute. The

reactions were excited at 485 nm and the emission signal was
measured at 518 nm.

Amplification with an anchor p1 primer (HIV12, table
1) was performed in combination with a standard p2 primer

5   (HIV2, table 1). The anchor primers consist of a 22nt anchor
sequence upstream of the T7-promoter sequence and 7nt
hybridizing sequence downstream of the T7-promoter sequence.
Amplification with standard p1 primer (HIV1, table 1) and
standard p2 primer (HIV2, table 1) was used as reference. The

10  results show that sensitivity of amplification with the
anchor primer is comparable to the reference primer (figure
2).


**EXAMPLE 2**

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<u>Amplification of HIV RNA with p1 primer with very short
hybridisation sequence</u>

Amplification with a p1 primer identical to HIV12,
but no anchor sequence (HIV11, table 1, figure 3), containing

20  only the 3' hybridizing target specific sequence of 7
nucleotides, was performed in combination with a standard p2
primer (HIV2, table 1). HIV1 and HIV12 (table 1) were used as
reference. Amplification was performed as described in
example 1. As shown in figure 4, no amplification was

25  observed with primer HIV11 (Fig. 4B), indicating that the
anchor sequence is required for efficient amplification. The
anchor is assumed to be necessary during the initiation of
the reaction and not in the cyclic phase. Therefore, addition
of a non-anchored primer can be of advantage during the

30  cyclic phase of the reaction. When using both the anchor
primer HIV12 and primer HIV11 in one and the same
amplification reaction, a slight improvement of the reaction
kinetics was observed (figure 4D).

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EXAMPLE 3


Amplification of HIV RNA with anchor p1 primers with
different anchor lengths

5          To investigate what minimum length of the anchor,
still results in a specific amplification, primers with
different anchor lengths were designed. Amplification with
anchor primers with different anchor lengths (deleted from
the 5' end) varying from 22 to 7 nucleotides (HIV12, 15, 16,
10   17, 20, 21, 22, table 1) was performed in combination with a
standard p2 primer (HIV2, table 1). The standard p1 primer
HIV1 (table 1) was used as reference. Amplification was
performed as described in example 1. Deletion up to 14
nucleotides still results in an efficient amplification as
15   compared to the references  (HIV1 standard primer and HIV12
anchor primer) (figure 5). However, anchor lengths below 14
nucleotides show very little or no amplification. The
hybridization temperature of 14 nucleotides or more is
expected to be higher then 41°C (amplification temperature).
20   Shorter anchors could have hybridization temperatures below
41°C, which may explain inefficient amplification.


EXAMPLE 4


25   Amplification of HIV RNA with anchor p1 primers using 2'-O-
Methyl modified nucleotides in anchor sequence
          In order to increase the binding efficiency of the
anchor, 2'-O-Methyl modified nucleotides are incorporated in
the anchor. Primers with anchor lengths of 14nt (HIV17 MET,
30   table 1), 12nt (HIV20 METa & b, table 1) , 9nt (HIV21 METa &
b, table 1) and 7nt (HIV22 METa & b) were used in
amplification. The expected melting temperature increase is
about 1.5°C per incorporated 2'-O-Methyl modified nucleotide.

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A standard p2 primer (HIV2, table 1) was used as reverse
primer. Amplification was performed as described in example
1. Using these anchor primers with 2'-O-Methyl modified
nucleotides clearly improves the sensitivity of the
5    amplification reaction (figures 6-8), in particular when
using anchors shorter then 14 nucleotides. (12 & 9nt anchors
HIV20 MET and HIV21 MET). 7nt anchor primers with 2'-O-Methyl
modified nucleotides (HIV22 MET) turn out to be too short
(result not shown). Probably, 7 nucleotide anchors, even with
10   2'-O-Methyl modified nucleotides, have hybridization
temperatures below  41°C, explaining inefficient
amplification.


     EXAMPLE 5

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     Amplification of HIV RNA with anchor p1 primers using LNA
     (Locked Nucleic Acid) nucleotides in anchor sequence
              In order to increase the binding efficiency of the
     anchor, LNA nucleotides are incorporated in the anchor. The
20   expected Tm increase is about 3-8°C per incorporated LNA
     nucleotide. Four different anchor p1 primers with anchor
     lengths of 7nt (HIV22 LNA 1t/m4, table 1) were used in
     amplification. All these anchor p1 primers were tested in
     combination with a standard p2 primer. Amplification was
25   performed as described in example 1. Compared to the 7nt DNA
     anchor primer HIV22, LNA anchor primers clearly results in an
     improved sensitivity of amplification (table 1) (figure 9).
     Compared to the 14 nt anchor primer HIV17 (table 1) or the
     standard p1 primer HIV1 (table 1), the performance of these
30   LNA anchor primers is less. The position of the LNA
     nucleotides in the anchor sequence seems to effect
     amplification efficiency as a better sensitivity is obtained

using the anchor primers HIV 22 LNA 3 and 4 (table 1)
compared to the primers HIV22 LNA1 and 2 (table 1).

**EXAMPLE 6**

Amplification of HIV RNA with anchor primers using PNA
(Peptide Nucleic Acid) in anchor sequence

   In order to increase the binding stability of the
anchor, PNA nucleotides are incorporated in the anchor.
Primers with anchor lengths of 14nt (HIV17 PNA, table 1),
12nt (HIV20 PNA, table 1), 9nt (HIV21 PNA, table 1) and 7nt
(HIV22 PNA, table 1) were used in amplification in
combination with a standard p2 primer (HIV2, table 1). All
the anchors contain complete PNA sequences. Amplification was
performed as described in example 1. Although a 10-fold
decrease in sensitivity was observed as compared to DNA
anchor primers, also the PNA-anchored primers were shown to
be functional in NASBA (figure 10).

**EXAMPLE 7**

Amplification of HIV RNA with anchor p2 (reverse) primers

   Amplification with anchor p2 primers (HIV27 and
HIV29, table 1) was performed in combination with a standard
p1 primer (HIV1, table 1). Standard p2 primers (HIV2 and
HIV26, table 1) were used as a reference in combination with
standard p1 primer (HIV1, table 1). Amplification was
performed as described in example 1. For both amplifications,
kinetics and amplification sensitivity was observed to be
comparable to the reference amplification (figure 11).

**EXAMPLE 8**

Amplification of HIV RNA with anchor p2 primers with
different anchor lengths

        Amplification with anchor p2 primers with different
anchor lengths varying from 22 to 14 nucleotides (HIV27, 31,
5    32, 33 and 29, table 1) was performed in combination with the
standard p1 primer (HIV1, table 1). Amplification was
performed as described in example 1. Shorten the anchor
sequence of the p2-primers to 14 nucleotides results in
comparable amplification efficiency as compared to the
10   reference (HIV27 anchor primer, table 1) (figure 12).


    EXAMPLE 9


Amplification of HIV RNA with anchor p1 primer and anchor p2
15   primer

        Amplification with anchor p1 primer (HIV17, table 1)
and anchor p2 primers (HIV27 and HIV29, table 1) resulted in
comparable amplification efficiency compared to the standard
primer set (HIV1/ HIV2, table 1) (figure 13). Amplification
20   was performed as described in example 1.


    EXAMPLE 10
Amplification of HCV RNA with anchor p1 primer

        Also for the amplification of HCV RNA a NASBA was
25   designed making use of an anchor p1 primer. Amplification
with anchor p1 primer (HCV13 or HCV 49, table 2) and a normal
p2 primer (HCV2, table 2) was performed. The anchor primers
consist of a 14 nt anchor sequence upstream of the T7-
promoter sequence and 7nt hybridizing sequence downstream of
30   the T7-promoter sequence. Amplification with standard p1
primer (HCV1, table 2) and standard p2 primer (HCV2, table 2)
was used as reference. Molecular beacon HCV-WT3 (table 2) is
used as probe and in vitro obtained HCV RNA transcript as

input. Amplification was performed as described in example 1
except that the denaturing step at 65°C was performed for 5
minutes instead of 2 minutes. Although differences in
sensitivity and kinetics are observed, the results show that
5    anchored primers can be used for amplification of HCV RNA
(figure 14).


**EXAMPLE 11**


10   Amplification of HCV RNA with anchor p1 primer, having a 3nt
gap between the binding site of the anchor and the 3'
hybridizing sequence

Amplification with anchor p1 primer (HCV22, table 2)
and a normal p2 primer (HCV2, table 2) was performed. In this
15   anchor, a 3 nt gap separates the binding site of the anchor
sequence from that of the 7nt 3' hybridizing sequence.
Amplification with anchor p1 primer HCV13 (table 2) and
standard p2 primer (HCV2, table 2) was used as reference.
Molecular beacon HCV-WT3 (table 2) is used as probe and in
20   vitro obtained HCV RNA transcript as input. Amplification was
performed as described in Example 10. The results show that a
3 nt gap can exist between the binding site of the anchor and
that of the 3' hybridizing sequence (figure 15), and that
these anchored primers can be used to bridge non-conserved
25   nucleotides in the primer-binding site.

## Table 1

| NR° | Sequence 5'-> 3' |
|---|---|
| | *Standard primers* |
| HIV1 (p1) | *AATTCTAATACGACTCACTATAGGG* **TGCTATGTCACTTCCCCTTGGTTCTCTCA** |
| HIV2 (p2) | **AGTGGGGGGACATCAAGCAGCCATGCAAA** |
| HIV26 (p2) | **AGTGGGGGGACATCAAGCAGC** |
| HIV11 | *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> **TCTCTCA** |
| | *Anchor p1 primers* |
| HIV12 | TGCTATGTCACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u>TCTCTCA |
| HIV15 | CTATGTCACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV16 | TGTCACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV17 | CACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV20 | CTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG_AAACGGGCACGAGC* TCTCTCA |
| HIV21 | CCCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV22 | CCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| | *Anchor p1 primers with 2'-O-Methyl nucleotides in anchor* |
| HIV17 MET | CACTT⸰CCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV20 METa | CTT⸰CCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV20 METb | ⸰TTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV21 METa | ⸰CCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u>TCTCTCA |
| HIV21 METb | ⸰CCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV22 METa | ⸰CCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV22 METb | ⸰CCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| | *Anchor p1 primers with LNA nucleotides in anchor* |
| HIV22 LNA1 | ⸰CCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV22 LNA2 | CC⸰TGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV22 LNA3 | ⸰CTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV22 LNA4 | ⸰C⸰TGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| | *Anchor p1 primers with PNA anchor* |
| HIV17 PNA | ⸰ACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV20 PNA | ⸰TTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG_AAACGGGCACGAGC* TCTCTCA |
| HIV21PNA | ⸰CCCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| HIV22 PNA | ⸰CCTTGGT *AATTCTAATACGACTCACTATAGGG* <u>AAACGGGCACGAGC</u> TCTCTCA |
| | *Anchor p2 primers* |
| HIV27 | AGTGGGGGGACATCAAGCAGCC <u>*GACTTCAGGACTTCAGG*</u> ATGCAAA |
| HIV31 | GGGGGACATCAAGCAGCC <u>*GACTTCAGGACTTCAGG*</u> ATGCAAA |
| HIV32 | GGGACATCAAGCAGCC <u>*GACTTCAGGACTTCAGG*</u> ATGCAAA |
| HIV33 | GACATCAAGCAGCC <u>*GACTTCAGGACTTCAGG*</u> ATGCAAA |
| HIV29 | AGTGGGGGGACATC <u>*GACTTCAGGACTTCAGG*</u> AAGCAGC |
| | *Molecular beacon for real time detection* |
| HIV MB WT | gcatgc **ATCAATGAGGAIGCTGCAGAITGGGA** gcatgc      (5' FAM / 3' dabcyl labeled) |

Target specific sequence in bold, T7 promoter sequence in italic, Y sequence (p1 primers) or X sequence (p2 primers) in italic and underlined, modified nucleotides in anchor with grey background

## Table 2

| NR° | Sequence 5'-> 3' | |
|-----|------------------|--|
| | *Standard primers* | |
| HCV1 (p1) | *AATTCTAATACGACTCACTATAGGG* **CAAGCACCCTATCAGGCAGTA** | |
| HCV2 (p2) | **GTCTAGCCATGGCGTTAGTA** | |
| | *Anchor p1 primers* | |
| HCV13 | **CAAGCACCCTATCA** *AATTCTAATACGACTCACTATAGGG* *AAGAGGGCACGAGC* **GGCAGTA** | |
| HCV22 | **TCGCAAGCACCCTA** *AATTCTAATACGACTCACTATAGGG* *AAGAGGGCACGAGC* **GGCAGTA** | |
| HCV49 | **CAAGCACCCTATCA** *AATTCTAATACGACTCACTATAGGG* *AAACGAGCACGAGC* **GGCAGTA** | |
| | *Molecular beacon for real time detection* | |
| HCV WT3 | gctagc **ATTTGGGCGTGCCCCCGCIAGA** gctagc | (5' FAM / 3' dabcyl labeled) |

Target specific sequence in bold, T7 promoter sequence in italic, Y sequence (p1 primers) or X sequence (p2 primers) in italic and underlined